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Lysosomal Enzyme Release: A Possible Mechanism of Action of Cobalt as an Erythropoietic Stimulant (38192)

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The role of the kidney in erythropoietin (ESF) production has been well established (1–4). ESF, a glycoprotein hormone, has been postulated to be produced enzymatically by the interaction of a renal erythropoietic factor (REF, erythrogenin), located in the lysosome-containing, light mitochondrial fraction of renal tissue, with an α-globulin (5–7).

Although the erythropoietic effects of cobalt have been demonstrated to be due to increased renal production of ESF (1–4, 8), the mechanism of action of cobalt is not yet clear. It has been postulated that cobalt stimulates the production of erythropoietin by causing a state of histotoxic (tissue) hypoxia in the kidney. DeDuve and others have shown that tissue ischemia is accompanied by labilization of lysosomal membranes with subsequent release of granule contents (9–11). In view of the reported presence of REF activity in lysosome-rich kidney fractions (12), perhaps a parallel could be drawn between the effects of ischemia and those of cobalt in terms of the capacity of cobalt to stimulate ESF production.

There have been a number of attempts to explain cobalt polycythemia in terms of an hypoxic mechanism. The purpose of our studies was to evaluate the effects of cobalt on renal tissue at the subcellular level. Cobalt, acting as an erythropoietic stimulant, is unique in that other metals with similar subcellular actions fail to elicit a polycythemia. Our goal was to correlate the cellular actions of cobalt with its capacity to stimulate red blood cell production.

Materials and Methods. Preparation of normal dialyzed serum (NRS). Male Sprague-Dawley rats (250–300 g) were anesthetized with ether and blood withdrawn from the abdominal aorta with a plastic syringe. Blood from 4–5 rats was pooled and allowed to clot for 24 hr at 4°. The serum was collected and dialyzed against 100 vol of 5×10^{-3} *M* EDTA (pH 7.0, 4°) for 24 hr. The serum was again dialyzed against 100 vol of distilled water (pH 7.0, 4°) for 24 hr and then frozen at −70° until used in the incubation studies.

Preparation of renal erythropoietic factor. Male Sprague-Dawley rats (250–350 g) were injected with cobaltous chloride hexahydrate (250 μmoles/kg, sc) and then sacrificed at 1,4,8,12,18,24,48, and 72 hrs after injection. At each time interval the rats, in groups of 6, were anesthetized and subsequently bled and nephrectomized. Plasma was prepared and frozen until used. The renal erythropoietic factor was prepared from the kidney tissue by a method described previously (12, 13).

Incubation procedure for REF and NRS. The reaction mixture consisted of equal volumes of the light mitochondrial extract (REF) and NRS. The pH was adjusted to 6.8 and the incubation carried out at 37° for 60 min in a shaking incubator. The reactions were stopped by immersing the reaction vessels in ice water. The REF-NRS mixtures were then administered to

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the ex-hypoxic polycythemic mice at the end of the incubation period.

The ex-hypoxic polycythemic mouse assay. HAM/ICR (CD-1) female Charles River mice (22–26 g) were made polycythemic according to a modification of the method of Cotes *et al.* (14) by exposure to 0.42 atmosphere for 14 days in a hypobaric chamber. The mice were removed from the chamber for 2 hr each day at which time they were given water and food. At the end of the 2 week period the mice were removed from the chamber and allowed to equilibrate at normal atmospheric pressure. On the fourth posthypoxic day the mice were injected ip with the test material to be assayed. Except for the REF-NRS mixtures (2.0 ml), all samples (1.0 ml) were injected in a divided dose sc on the fourth and fifth posthypoxic days. On the sixth post-hypoxic day each mouse received 0.5 μCi of ^{59}Fe citrate via the tail vein. On the eighth posthypoxic day the mice were bled by cardiac puncture, blood counted in a Packard Auto-Gamma spectrometer and the percent ^{59}Fe incorporation in red cells determined. The percent iron incorporation was converted to units of erythropoietin from a 4 point log dose-response curve for the International Reference Preparation (IRP) of erythropoietin.

Assay of lysosome membrane integrity. Male Sprague-Dawley rats (200–250 g) received a subcutaneous injection of cobaltous chloride, ferrous sulfate, manganous sulfate, or nickel chloride in doses that have been employed previously to evaluate the erythropoietic effects of these metals (8). The animals were anesthetized at 6 and 12 hrs after treatment and subsequently bled and nephrectomized. Plasma was obtained and frozen ($-10°$) until used. A light mitochondrial fraction (LMF) was prepared from the kidneys of rats treated with cobalt for 12 hr according to the method described previously.

Labilization or stabilization of lysosomes in the LMF by cobalt was ascertained by determining the osmotic release of the lysosomal marker enzyme aryl sulfatase by a modification of procedures described previously (15). Briefly, a suspension of the LMF in 0.45 *M* sucrose–0.04 *M* Tris acetate buffer, pH 7.4 was warmed to 25° for 5 min and 0.2 ml aliquots (equivalent to 0.16 grams of kidney) were added to glass tubes containing 2.0 ml of 0.18 *M* sucrose–0.04 *M* Tris acetate buffer, pH 7.4 at 25°, and the tubes were incubated at 37° for 30 min in a Dubnoff metabolic shaker set at 150 agitation cycles/min. The incubation was terminated by high speed centrifugation (27,000*g* for 15 min at 4°) after transfer of the samples to 15 ml polyethylene tubes. The high centrifugal force was employed to sediment much of the suspended particles, thereby rendering the clear supernatant fractions more suitable for subsequent measurements of enzyme activity. Supernatants were decanted into small glass tubes and maintained at 4° until assayed for lysosomal enzyme activity. Aryl sulfatase activity was measured using 2-hydroxy-5-nitrophenyl sulfate as substrate, according to the method described by Roy (16).

Plasma enzyme determinations. Plasmas obtained from rats treated with cobalt (6 and 12 hrs) were thawed and warmed to 25°. Aliquots (0.4 ml) of plasma were assayed for β-glucuronidase activity according to the method of Gianetto and DeDuve (17). The formation of phenolphthalein from phenolphthalein glucuronide was measured. Acid phosphatase activity was determined by the method of Torriani (18). The formation of *p*-nitrophenol from *p*-nitrophenyl phosphate was measured. Plasma obtained from rats that were bilaterally nephrectomized (6 and 12 hrs) and rats that received cobalt (250 μmoles/kg, sc) immediately following nephrectomy for 6 and 12 hrs were evaluated for enzyme activity.

Results. The data in Table I demonstrate the effects of cobalt on REF activity and ESF production in the rat. Cobalt caused significant ($P < 0.05$) stimulation of REF activity at 8, 12 and 18 hrs after cobalt treatment as compared to control values. REF incubated with saline at 37° for 60 min (pH 6.8) failed to demonstrate erythropoietic activity at any time interval. We have shown previously that the activity

TABLE I. Renal Erythropoietic Factor Activity and Erythropoietin Production in Rats Following Cobalt Administration.

Treatment time (hrs)	No. of mice	Units ESF generated/2 ml of LME-NRS reaction mixture[a]	Units ESF/ml plasma
0	20	0.050 ± .006[b]	0.069 ± .010
1	20	0.055 ± .007	0.065 ± .006
4	19	0.062 ± .003	0.085 ± .014
8	20	0.125 ± .004[c]	1.700 ± .230[c]
12	18	0.160 ± .040[c]	2.100 ± .600[c]
18	20	0.110 ± .002[c]	0.600 ± .050[c]
24	17	0.065 ± .020	0.300 ± .031[c]
48	20	0.045 ± .001	0.092 ± .020
72	18	0.030 ± .003	0.060 ± .034

[a] Equal vol of a light mitochondrial (hypotonic, water) extract (LME) and normal dialyzed rat serum (NRS) were incubated at 37°, for 60 min, pH 6.8. The reaction mixtures in 2 ml aliquots were evaluated for erythropoietic activity in the ex-hypoxic polycythemic mouse assay.

[b] Each value represents the mean ± S.E.M.

[c] Significantly different ($P < .05$) from control.

generated by the REF-NRS reaction mixture can be blocked by antibody to erythropoietin (12).

Plasma ESF levels (Table I) were significantly ($P < 0.05$) elevated above control values at the 8, 12, 18 and 24 hrs intervals following cobalt administration. When a mixture of NRS and saline was injected into the assay mice no significant erythropoietic activity was detected.

Table II illustrates the changes in lysosomal enzyme release that occurred when suspensions of the LMF were incubated at 37°. At the commencement of the incubation period, enzyme (aryl sulfatase) activity was found to be latent to the extent of 82–85%. Cobalt provoked a significant ($P < 0.05$) increase in the release of aryl sulfatase from the lysosome fraction, when compared to control values. Iron, manganese, and nickel had no effect on lysosomal enzyme release.

TABLE II. *In Vivo* Effect of Various Metallic Ions on Lysosomal Membrane Integrity in the Rat Kidney.

Agent	Dose (μM/kg)	Aryl sulfatase release (% total activity) incubation time (min)[b] 0	30	60	90
Control	—	15 ± 1.6[c]	22 ± 2.3	22 ± 2.4	23 ± 2.7
Cobaltous Chloride[a]	250	18 ± 2.0	42 ± 5.1[d]	43 ± 4.2[d]	48 ± 3.6[d]
Ferrous Sulfate	200	13 ± 1.4	18 ± 2.4	19 ± 1.7	18 ± 2.3
Manganous Sulfate	600	12 ± 1.5	19 ± 2.1	16 ± 2.0	17 ± 3.2
Nickel Chloride	400	18 ± 2.4	19 ± 1.1	23 ± 2.3	24 ± 2.0

[a] Each of the metals tested was administered in the form of a subcutaneous injection.

[b] INCUBATION MEDIUM: 0.18 sucrose–0.04 M Tris acetate, pH 7.4. Incubations were conducted at 37° for 30 min.

[c] Data represent the mean ± S.E.M. from 4–5 separate experiments. Actual extinction values for the control (LMF suspension incubated without metals for 0, 30, 60 and 90 min, respectively) were 0.01–0.03, 0.06–0.08, 0.44–0.51 and 0.60–0.65. Extinction values were determined at 510 nm.

[d] Significantly different ($P < 0.05$) from control.

In vivo labilization of lysosomes is paralleled by elevated plasma levels of the enzymes associated with these granules (19–20). Animals that were injected with cobalt demonstrated significantly ($P < 0.05$) increased plasma titers of the lysosomal hydrolases β-glucuronidase and acid phosphatase (Fig. 1) at 6 and 12 hrs following cobalt treatment. A temporal relationship existed between the labilizing effect of cobalt *in vivo* and the appearance of elevated lysosomal enzyme activities in plasma. In addition, the appearance of lysosomal hydrolases in the plasma of rats treated with cobalt (6–12 hr) correlates with the observed increase in REF activity (8–18 hr) and ESF production (8–24 hr) following cobalt administration, both of which are demonstrated in Table I. No significant changes in plasma levels of acid phosphatase or β-glucuronidase were observed in rats following nephrectomy or nephrectomy plus cobalt treatment.

Discussion. We report here that increased renal erythropoietic factor activity and erythropoietin production are seen in the rat following cobalt administration. Based on the hypothesis that upon receiving an hypoxic stimulus (e.g., cobalt) the kidney generates/activates a factor (REF) which is released into the circulation and acts upon a plasma protein to generate erythropoietin, we attempted to investigate more closely the mode of action of cobalt as an erythropoietic stimulus.

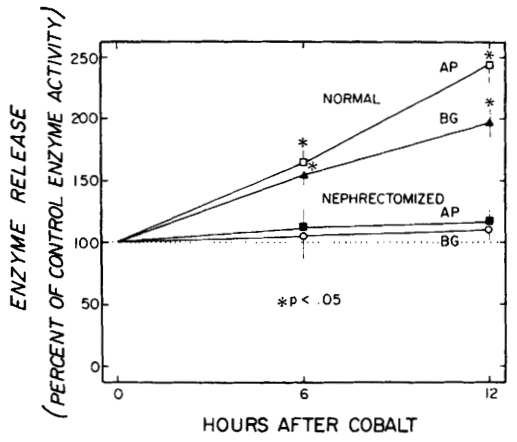

FIG. 1. Effect of cobalt on plasma levels of acid phosphatase (AP) and β-glucuronidase (BG) in normal and nephrectomized rats. Each value represents the mean ± S.E.M. from 5 separate experiments. Control enzyme activity was set at 100%. Asterisks indicate values which are significantly ($P < 0.05$) different from the control value.

The mechanism of cobalt polycythemia is not completely understood. Low doses of cobalt have been reported to inhibit tissue respiration *in vitro* (21). At the subcellular level, cobalt was reported to inhibit several oxidative enzymes in the rat kidney (22). This effect was accompanied by a decrease in respiration and a depression of oxidative phosphorylation in the renal tissue (23). Attributing the erythropoietic stimulation action of cobalt to depression of oxidative phosphorylation appears rather paradoxical in light of the findings that other metals with similar actions on cellular respiration (i.e., cyanide and arsenite) elicit negligible effects on erythrocytosis.

In vitro studies performed in our laboratory indicate that cobalt (10^{-3}–10^{-8} *M*) has no effect on lysosomal membrane integrity *in vitro*. Similarly, other ions studied (iron, manganese, nickel), which are structurally similar to cobalt, do not affect the integrity of lysosome membranes. However, we report here that cobalt, but not the other metals studied, provokes labilization of lysosomes *in vivo*. Moreover, elevated plasma levels of lysosomal marker enzymes parallel the cobalt-induced lysosome labilization *in vivo*. We have also shown that the effects of cobalt on lysosomal membrane integrity and plasma levels of lysosomal hydrolases occur during time intervals when cobalt demonstrates a marked effect on REF activity and plasma levels of ESF. It is interesting to note that Harvey *et al.* (24) have demonstrated significant REF activity in a lysosome-rich kidney fraction taken from hypoxic rats. Furthermore, Libbin and Gordon (25) have recently reported that various subfractions of the light mitochondrial fraction prepared from the kidneys of rats, which were pretreated with Triton WR-1339 and then subjected to an hypoxic stimulus, possessed significant amounts of REF activity as well as lysosomal enzyme activity. These reports sup-

port our hypothesis that REF activity is associated with the lysosomal granules of rat kidney. The additional findings, in the present report, that nephrectomy abolishes the cobalt-induced elevation of plasma lysosomal enzyme activity indicate that the action of cobalt *in vivo* on lysosome membrane integrity is due to an effect on renal cells.

The hypothesis has been set forth that the kidney, in response to an hypoxic stimulus such an cobalt, produces a renal erythropoietic factor (REF) that is subsequently released into the circulation, where it interacts with a plasma protein substrate resulting in the generation of erythropoietin (5–7). The data in this report suggest that cobalt, acting as a stimulus for ESF production, provokes the release of lysosomal enzymes from the kidney and the subsequent release of REF. The mechanism of lysosomal enzyme release by cobalt might in part be due to lysosome membrane labilization, an event provoked, perhaps, by a decrease in intracellular pH (26). In this way, cobalt-induced renal histotoxic hypoxia might be analogous to generalized tissue ischemia. Hypoxia or ischemia of certain renal cells, accompanied by concomitant lysosomal labilization, could result in the release of REF into the circulation, with the subsequent formation of ESF and, ultimately, red blood cell production.

Summary. Cobalt produced a significant increase in renal erythropoietic factor activity in the kidney and plasma levels of erythropoietin, which reached peak activity 12 hr after treatment. The studies reported here indicate also that cobalt caused the release of lysosomal enzymes into the blood *in vivo*. Furthermore, animals treated with cobalt showed increased plasma titers of lysosomal marker enzymes (β-glucuronidase and acid phosphatase) at a time when REF activity and plasma ESF levels were approaching a maximum. Metallic ions similar in structure to cobalt (iron, manganese, nickel) did not affect the integrity of lysosomal membranes *in vivo*. In addition, neither cobalt nor any of the metals tested altered lysosome membrane integrity *in vitro*.

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